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In vitro asymbiotic germination, seedling formation and tuberization of *Orchis sancta*

Orchis sancta'nın *in vitro* asimbiyotik çimlenmesi, fide oluşumu ve tüberizasyonu

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ABSTRACT

Plant tissue culture techniques have recently become attractive tools for propagating rare and endangered species and producing their economically important natural products. Here, we introduce a protocol for an effective, fast and reliable production route of plantlets and tubers from salep orchid, *Orchis sancta*, a native orchid species growing wild in Turkey. In the course of this study, the efficacy of basal media and plant growth regulators on asymbiotic germination of mature seed, seedling formation, tuberization and glucomannan contents of tubers were assessed. Amongst all basal media evaluated, Orchimax including activated charcoal gave the best germination rate (62.46%). Supplementing this medium with 1.0 mg/L zeatin resulted in the highest germination rate (81.18%). Lowest thidiazuron concentration (0.25 mg/L) was very effective on shoot elongation while kinetin (0.5 and 1.0 mg/L) promoted leaf formation. The aforesaid basal medium with IBA favored root extension (31.16 mm) and root formation (4.5 roots per plantlet) at 1.0 mg/L and 2.0 mg/L IBA, respectively. Cytokinins, zeatin (2.0 mg/L) in particular, promoted tuber formation and glucomannan accumulation. Plantlets were successfully adapted to soil step by step.

ÖZ

Bitki doku kültürü teknikleri, son zamanlarda, nadir ve nesli tükenmekte olan türlerin çoğaltılması ve ekonomik açıdan önemli doğal ürünlerinin üretilmesi için ilgi çekici araçlar haline gelmiştir. Bu çalışmada, Türkiye'de doğal olarak yetişen yerli bir sahleb orkidesi türü olan *Orchis sancta*'nın fide ve yumrularının *in vitro* koşullarda etkin, hızlı ve güvenilir bir üretim protokolü oluşturulmuştur. Buna istinaden, olgun tohumların asimbiyotik çimlenmesi, fide oluşumu, yumru oluşumu ve yumru köklerin glukomannan içerikleri üzerine bazal ortam ve bitki büyüme düzenleyicilerinin etkinlikleri değerlendirilmiştir. Değerlendirilen tüm bazal ortamlar arasında aktif kömür içeren Orchimax en iyi çimlenme oranını (%62.46) verdi. Bu ortamın 1.0 mg/L zeatin ile desteklenmesi en yüksek çimlenme oranı (%81.18) ile sonuçlanmıştır. En düşük thidiazuron konsantrasyonunun (0.25 mg/L) sürgün uzaması üzerinde, kinetin'in (0.5 ve 1.0 mg/L) ise yaprak oluşumu üzerinde daha etkili olduğu belirlenmiştir. Yine aynı bazal besi ortamının 1.0 mg/L ve 2.0 mg/L IBA ile desteklenmesiyle sırasıyla kök uzamasını (31.16 mm) ve kök oluşumunu (fide başına 4.5 adet kök) daha fazla teşvik etmiştir. Sitokininlerin, özelliklede 2.0 mg/L zeatin'in, yumru oluşumunu ve yumrulardaki glukomannan birikimininde daha etkili olduğu tespit edilmiştir. *İn vitro* koşullarda üretilen fideler kademeli bir şekilde toprak koşullarına adapte edilmiştir.

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1. INTRODUCTION

The Orchidaceae family, which includes ornamental plants with fascinating flowers, also contain salep plants, which are used in salep production. The

underground organs of the orchids are tubers or rhizomes and the formers are generally found as couples (sometimes more than two) and they are collected for the production of salep. The primary components of these orchid tubers are glucomannan, starch, sugar and nitrogenous compounds (Sezik, 1984).

Due to their economic values, these orchids are constantly threatened by people. Many of these plants that grow naturally in Turkey are mostly endemic and face extinction due to uncontrolled collection. In situ germination of their seeds are extremely difficult and takes a long time and they are not applicable for agricultural purposes. The most ideal, fast and controlled reproduction procedure that can be applied for the production of these species is "propagating plants or products using tissue culture techniques". Nevertheless, such approaches are comparably new and therefore only handful information is available in the literature (Sgarbi et al., 2009; Bektaş et al., 2013; Bektaş & Sökmen, 2016). Also, it should be born in mind that in vitro production methods applied may vary from one species to another.

This study aimed to develop effective production protocols for *Orchis sancta* L. by using the current plant tissue culture methods. A complete micropropagation method has been proposed for this purpose, including *in vitro* plantlet and tuber formation and acclimatization to the soil conditions.

2. MATERIALS AND METHODS

2.1. Surface disinfection and seed germination

Seeds of *O. sancta* were supplied from Aegean Agricultural Research Institute (İzmir, Turkey) and used for culture initiation. For the surface sterilization of the seeds, a method was followed as described elsewhere (Chua et al., 2012). Four basal media are specified in Table 1 each being supplemented with six plant growth regulators (PGRs; IAA, IBA, 2,4-D, ZEA, 6-BA and TDZ) at the concentrations of 0.25, 0.5, 1.0 and 2.0 mg/L were used for preliminary experiments concerning *in vitro* germination. Results obtained from media without PGR were considered as a control. Cultures were set at 23±2°C, 16/8 hours photoperiod regime and 32 µmol s^{-1} m^2 light density for 3 months. At the end of the 2nd month seed germination rates (percentages), and the 3rd month protocorm formation rates (percentages) were recorded.

2.2. Shoot formation

The aforesaid PGRs (with different concentrations) were also evaluated in terms of their possible effects on shoot elongation, leaf and root formation of *O. sancta* seedlings grown under in vitro conditions. Protocorms formed by germinating seeds were used as explants. The shoot elongation (after 2 months) and the number of leaves, number and elongation of roots (after 3 months) were determined. Each experiment was performed with 20 samples as triplicates.

2.3. Adaptation of seedlings to soil conditions

Seedlings, which came to the stage of transferring to the soil, were cleaned in such a way that no residue of the nutrient medium could remain, and they were planted in culture dishes containing sterile peat. Culture dishes were incubated with their lids closed for 30 days to eliminate the risk of dehydration and possible contamination. At the end of this period, the lids were opened and the growth of the seedlings continued this wise until the end of the 3rd month. At the end of this incubation period, the viability rates (survivability) of the seedlings were determined.

2.4. Detection of glucomannan content

The glucomannan contents of *O. sancta* tubers were defined by the procedure presented in the literature (Stewart & Kane, 2006). After the tubers were dried under suitable conditions, they were pulverized with a grinder.

Solutions including of glucose (0.0, 16.0, 32.0, 48.0, 64.0, 80.0 µg/mL), were settled into the tubes and last volume was finished to 2 mL. 5% phenol reagent (1000 µL) was appended to the test tubes. 5.0 mL condensed sulfuric acid (95-97%) was mixed and then incubated at room temperature (10 min) and 25°C (20 min), respectively. Absorbance was evaluated (490 nm) and the amount of glucose curve against absorbance was drawn. The same method was followed for the mannose calibration curve. 2 mL of dH_2O were used as blank.

50 mg of powdered tuber sample was mixed on a magnetic stirrer with dH_2O (40 mL) at ambient

conditions for 4 hours. After 4 hours of mixing, the volume was completed to 50 mL and centrifuged (25 °C, 4000xg, 40 min). 46 mL of dH_2O were added to the 4 mL of supernatant to adjust the final volume to 50. At the end of these processes, glucomannan amounts were calculated by using the phenol-sulfuric acid method given above.

2.5. Statistical analysis

Statistical analysis was done using the SPSS program, Version 17.0 (SPSS Inc., Chicago, IL, USA). Variance analysis was performed to determine the significance of the means. The significance of the differences between these means was identified using the One-Way ANOVA Test and Duncan multiple range test at 0.05 probability.

3. RESULTS AND DISCUSSIONS

The mature seeds of *O. sancta* were germinated at higher rates and shorter time in all tested basal culture media than in their natural environment (Table 1). The highest germination rate (62.46%) and shortest germination time (15 days) were observed in the OM+ culture medium. KCM and LM showed similar effects on germination rates and germination periods with the lowest results. In addition, the protocorm formation ability of germinated seeds in these basal culture media was determined. Accordingly, the highest protocorm formation rate was observed in the OM+ culture medium (73.42%). This rate is regarded as an indicator, exhibiting how much-germinated seeds will turn into plants. So long as the conditions are suitable, each protocorm could create a new plant (rarely more than one). In natural habitats, a very small amount of orchid seeds could germinate, if suitable conditions were provided. Plant tissue culture media play an important role in ensuring these environmental conditions. Bektaş et al. (2013) and Bektaş and Sökmen (2016) reported that the Orchimax including activated charcoal culture medium (OM+) was the most effective basal culture media on the germination of *Orchis coriophora* and *Serapais vomeracea* mature seeds, similar to our results.

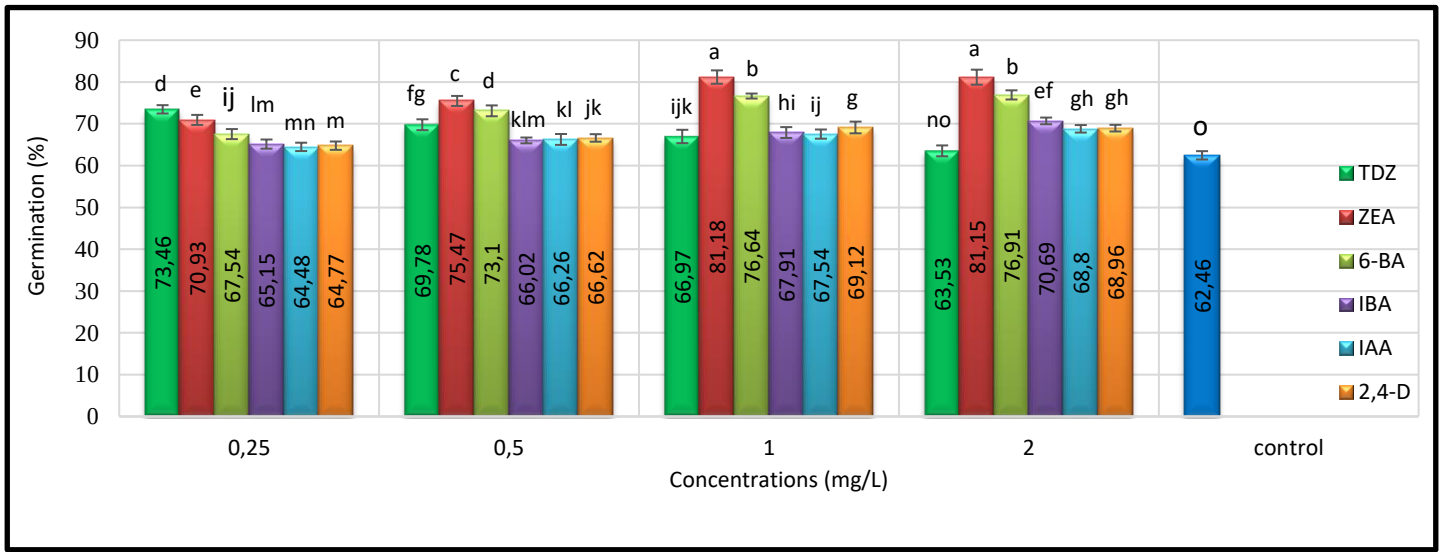

Figure 1. Efficacy of PGRs on the germination of *Orchis sancta* seeds on Orchimax containing activated charcoal. The values with different letters are significantly different (P<0.05) and standard deviations were shown with bars

Orchimax culture media (both including and without activated charcoal) contain an organic nitrogen source (tryptone) which has an important effect on orchid seed germination. The existence of an organic nitrogen source in culture media positively affects the germination of orchid seeds (Van Waes & Debergh, 1986; Bektaş & Sökmen, 2016) whereas inorganic nitrogen inhibits the orchid seed germination (Paek & Murthy, 1977; Malmgren, 1992; Vesjadova, 2006). Unlike the OM medium, the OM + containing 2.0 mg/L of activated charcoal promoted germination as emphasized by Paek and Murthy (1977). These researchers reported that activated charcoal promotes germination and rooting by providing a dark

environment. Additionally, the addition of activated charcoal causes an increase in medium pH, then keeps the pH constant, and consequently promotes growth by elevating nitrogen uptake (Black et al., 1974).

Table 1. Effects of media on the germination and protocorm formation parameters

Media	Germination (%)	Protocorm Formation (%)	Germination Time (day)
KC	26.36±1.3c	51.95±1.4d	45
LM	26.87±1.4c	52.55±0.9c	45
OM-	39.82±1.5b	63.2±0.8b	25
OM+	62.46±1.0a	73.42±0.8a	15

OM-: Orchimax with no activated charcoal, **KCM:** Knudson C, **LM:** Lindemann medium, **OM+:** Orchimax containing activated charcoal. The values with different letters in a column are significantly different (P<0.05). Means are given with their standard deviations

Apart from the basal media, the effects of cytokinins and auxins on *in vitro* germination of *O. sancta* were also determined (Figure 1). The maximum germination rate was monitored in OM+ media comprising 1.0 mg/L of ZEA as 81.18 %. As a result, cytokinins were proven to be more effective in the germination of *O. sancta* seeds. Cytokinins have been reported as PGRs with the most favorable effect on the germination of terrestrial orchid seeds (Bektaş et al., 2013). Cytokinins have also been reported to promote seed germination by reducing or removing the inhibitory effect of abscisic acid on germination (Crafts & Miller, 1974). It is a well-known fact that orchids form a symbiotic relationship with mycorrhizal fungi to germinate in the natural environment. As reported before, symbiosis with certain fungi provide cytokinin for seed germination (Gaspar et al., 1996).

Increased concentrations of ZEA, IBA, IAA and 2,4-D exerted positive effects on protocorm formation (Figure 2), whereas no correlation was available for both TDZ and IBA. The highest protocorm formation (83.14%) was observed when OM+ basal medium was supplemented with 2.0 mg/L ZEA. This finding is similar to our previous report (Bektaş et al., 2013).

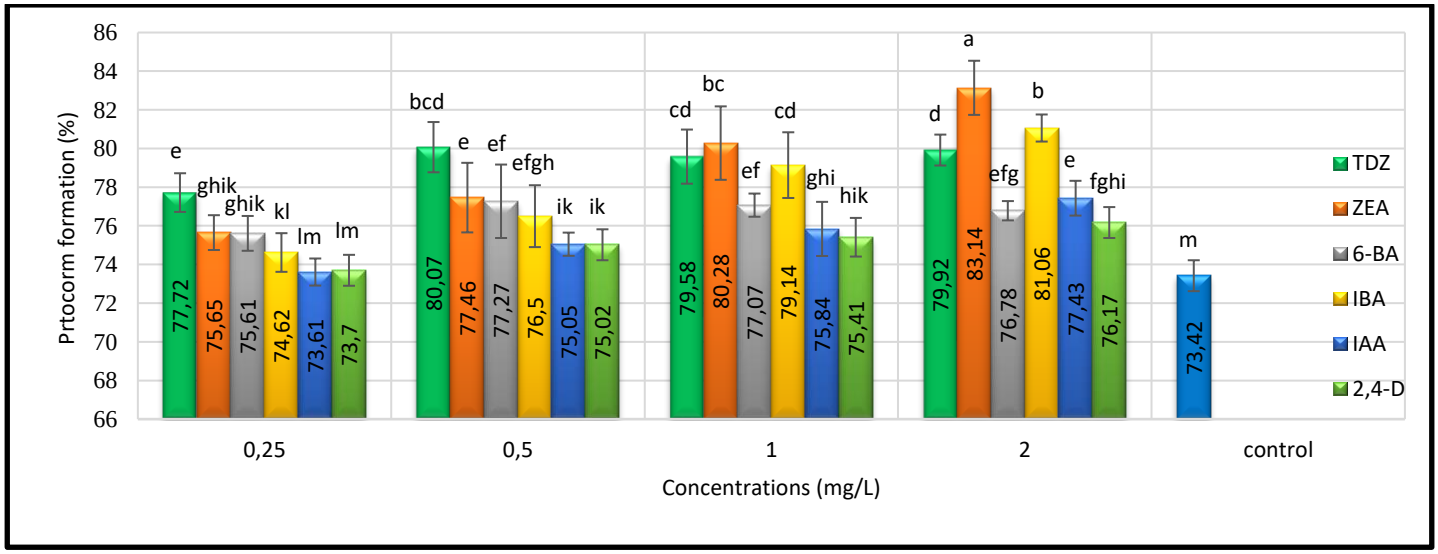

Figure 2. Efficacy of PGRs on protocorm formation of *Orchis sancta* on Orchimax including activated charcoal medium. The values with different letters are significantly different (P<0.05) and standard deviations were shown with bars

Plant growth regulators are the most commonly used chemicals to direct the development of explants in basal culture media. Auxins and cytokinins are the most commonly used plant growth regulators that play active roles in the differentiation of plant tissues. However, this may vary the species studied. Effects of cytokinins (Table 2) and auxins (Table 3) with various concentrations on shoot elongation, leaf formation, rooting and root elongation were individually determined in our present study.

Table 2. Impacts of cytokinins on the growth parameters of *Orchis sancta*

PGRs	Concentrations (mg/L)	Shoot elongation (mm)	Root length (mm)	Root number	Leaf number	Tuber formation	Glucomannan content (%)
TDZ	**0.25**	53.24±1.8 a	24.01±0.6b	2.8 defg	2.93abc	40.5	25.2±0.4
	0.5	50.63±0.9b	22.28±0.9c	2.8 defg	2.85bc	28.7	19.0±0.4
	1.0	44.81± 4.2c	17.95±0.6e	2.1 hı	2.78c	28.5	14.8±0.4
	2.0	44.03± 3.9c	12.75±0.6g	2.0 ı	278c	27.9	15.7±0.6
ZEA	**0.25**	30.88±1.8gh	28.86±0.7a	3.7 b	3.20abc	-*	-*
	0.5	35.52± 0.7f	23.65±0.9b	3.3 bcd	3.20abc	63.1	20.0±0.5
	1.0	39.86 ±2e	19.29±1.5d	3.2 cde	3.30abc	66.9	22.8±0.7
	2.0	42.75± 3.9d	17.21±0.8e	2.9 def	3.30abc	67.5	28.0±1.7
KIN	**0.25**	30.83±1.2gh	10.05±0.4jk	3.5 bc	3.46a	36.1	22.6±1.3
	0.5	32.24± 0.9g	11.55±0.8hı	3.5 bc	3.53a	36.0	26.2±1.1
	1.0	27.31± 0.8ıj	10.20±0.8jk	3.0 def	3.53a	40.5	20.3±0.7
	2.0	24.81±1.8k	9.62±0.7k	2.3 ghı	3.40ab	27.5	14.0±0.7
6-BA	**0.25**	26.57±2jk	12.63±0.8gh	3.0 def	3.10abc	-	-
	0.5	29.15±2hı	13.41±0.4 g	2.8 defg	3.10abc	-	-
	1.0	30.74±2.4gh	13.86±0.5 g	2.7 efg	3.20abc	-	-
	2.0	31.31±2.1gh	15.31±0.9f	2.5fgh	3.10abc	50.2	16.7±0.9
2-iP	**0.25**	24.78± 2.4k	9.56±0.7k	4.2 a	3.20abc	40.5	14.1±0.4
	0.5	26.96±1.6ı	9.95±0.4jk	3.7 b	3.20abc	35.1	15.6±0.7
	1.0	28.00±1.0ıj	10.83±0.3ıjk	2.8 defg	3.20abc	20.4	17.0±0.3
	2.0	31.20±1.2gh	11.13±1.1ıj	2.2 hı	3.30abc	19.3	21.3±0.9
Control		20.44±0.8l	8.31±0.5 l	2.0 ı	2.70c	-	-

OM+ free of PGRs was used as control. Length of shoot and number of leaves (60 days). Length and number of roots (3 months) and tuberization rates (6 months) were calculated days after planting of the protocorms on media. The values with different letters in a column are significantly different (P<0.05). Means were given with their standard deviations. *Tuberization was not observed

Firstly, TDZ, a cytokinin-like PGR, was more effective on shoot elongation as all TDZ concentrations applied better shoot elongation compared to the other PGRs studied. The highest shoot elongation was measured as 53.24 mm in OM+ with 0.25 mg/L TDZ. Secondly, the average numbers of leaves were calculated from the plantlets being cultured for 90 days. Accordingly, plantlets grown in aforesaid medium supplemented with 0.5 and 1.0 mg / L KIN gave the highest leaf formation with an average of 3.53 leaves. Thirdly, in terms of root formation, medium containing 1.0 mg/L IBA was found to be the most suitable medium for root extension. The average root elongation was calculated as 31.16 mm. The highest root formation (root number) was 4.5 in the presence of 2.0 mg/L IBA. Previous studies report that stimulation of shoot formation and inhibition of leaf abscission are among the physiological effects of cytokinin group (Skoog & Miller, 1957; Haberer & Kieber, 2002). In general, 1.0 to 2.0 mg/L of cytokinins are appropriate for shoot formation in most experiments, and higher concentrations tend to increase adventitious shoot formation (Werbrouck & Debergh, 1994; Aktar et al., 2007). In literature, some reports emphasize that auxins should be added to the culture media for root induction (Pedroso & Pais, 1992; Diaz & Alvarez, 2009).

Table 3. Impacts of auxins on the growth parameters of *Orchis sancta*

PGRs	Concentrations (mg/L)	Shoot elongation (mm)	Root length (mm)	Root number	Leaf number	Tuber formation	Glucomannan content (%)
IBA	**0.25**	31.76±1.3d	20.68±1.1g	4.1a	3.2abc	-	-
	0.5	38.98±2.1a	25.60±0.7de	4.3a	3.4ab	15.8	12.6±0.7
	1.0	37.07±1.8b	31.16±0.6a	4.4a	3.4ab	16.9	13.2±13.2
	2.0	35.06±1.4c	29.69±0.7b	4.5a	3.5a	16.8	15.0±0.4
IAA	**0.25**	29.83±1.6e	19.07±0.9h	3.0cd	3.0bcd	-	-
	0.5	32.38±1.2d	23.26±0.7f	3.2bcd	3.1abcd	-	-
	1.0	34.57±1.6c	26.77±0.7c	3.4bc	3.2abc	18.1	11.4±0.1
	2.0	34.58±1.3c	29.08±0.8b	3.5b	3.2abc	16.4	14.8±0.7
2,4-D	**0.25**	23.05±0.7g	15.34±1.3i	2.8d	2.7d	-	-
	0.5	24.28±0.6f	19.59±1.3h	2.8d	2.8cd	-	-
	1.0	25.26±0.7f	25.25±1.1e	3.1bcd	2.8cd	-	-
	2.0	24.38±0.7f	26.02±0.5d	3.4bc	3.1cd	-	-
Control		20.44±0.8h	8.31±0.5j	2.0 d	2.70d	-	-

OM+ free of PGRs was used as control. Length of shoot and number of leaves (60 days). Length and number of roots (3 months) and tuberization rates (6 months) were calculated days after planting of the protocorms on media. The values with different letters in a column are significantly different (P<0.05). Means were given with their standard deviations. * Tuberization was not observed

The effects of the auxins and cytokinins on the tuberization as well as their glucomannan contents were presented in Tables 2 and 3. *In vitro* production of *O. sancta* tubers took 6 months (Figure 3), starting from when protocorms were set to be cultured on OM+ medium. Cytokinins were found to be more effective on tuber formation and glucomannan accumulation as the highest tuber formation rate was determined in medium including 2.0 mg/L ZEA with a value of 67.5%. We also found that glucomannan contents in tubers were also at the highest level when aforesaid PGR is used, which is estimated as 28.0%. This value is comparable with those grown in the natural environment where the glucomannan content was 21.1%. Furthermore, the amounts of glucomannan contents of tuber grown on media supplemented with TDZ (0.25 mg/L) and KIN (0.5 mg/L) are also close to ZEA with values of 25.2% and 26.2%, respectively. This finding implies that TDZ, KIN and ZEA are all equally effective on glucomannan formation. On the other hand, none of the 2,4-D concentrations employed was effective on tuber formation. Pedroso and Pais reported that zeatin was the most effective PGR in minituber formation *Orchis papilionacea*. Cytokinins and gibberellins are the most important plant growth regulators managing tuber formation (Jackson, 1999; Sarkar, 2008). Furthermore, it has also been reported that gibberellins inhibit tuber formation, while cytokinins stimulate by triggering cell division (Vreugdenhil & Sergeeva, 1999; Fernie & Willmitzer, 2001).

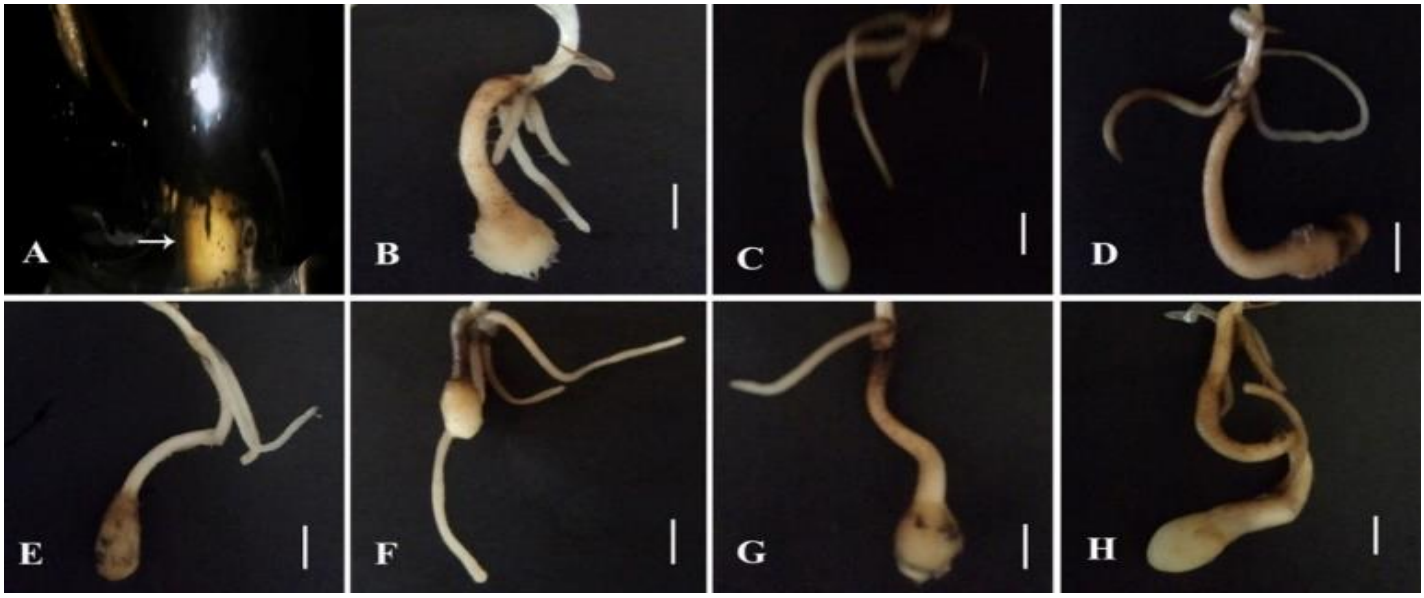

Figure 3. Pictures of *Orchis sancta* tubers growing in different media **A:** Picture of the tuber in OM+ medium, **B:** 0.25 mg/L TDZ, **C:** 2.0 mg/L IBA, **D:** 2.0 mg/L 2-iP, **E:** 2.0 mg/L 6- BA, **F:** 2.0 mg/L IAA, **G:** 0.5 mg/L KIN, **H:** 2.0 mg/L ZEA

The adaptation procedure of *O. sancta* plantlets to the soil condition has been carried out gradually. The plantlets were transferred to the sterile peat medium in the first stage and then successfully adhered to the soil conditions (Figure 4). This gradual adaptation process, which was previously carried out with *Serapias vomeracea*, may give fruitful results and could be applied to other orchid species (Bektaş et al., 2013).

Figure 4. Seedling development of *Orchis sancta* after 2 months of planting in sterilized peat medium

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REFERENCES

Aktar S, Nasiruddin KM, Huq H (2007). *In vitro* root formation in dendrobium orchid plantlets with IBA. *Journal of Agriculture and Rural Development* 5: 48-51.

Bektaş E, Cüce M, Sökmen A (2013). *In vitro* germination, protocorm formation and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. *Turkish Journal of Botany* 37: 336-342.

Bektaş E, Sökmen A (2016). *In vitro* seed germination, plantlet growth, tuberization and synthetic seed production of *Serapias vomeracea* (Burm. F.) Briq. *Turkish Journal of Botany* 40: 584-594.

Black M, Bewley JD, Fountain D (1974). Lettuce seed germination and cytokinins: their entry and formation. *Planta* 117: 145-152.

Chua M, Chan K, Hocking TJ, Williams PA, Perry CJ, Baldwin TC (2012). Methodologies for the extraction and analysis of konjac glucomannan from corms of *Amorphophallus konjac* K. Koch. *Carbohydrate Polymers* 87: 2202-2210.

Crafts CB, Miller CO (1974). Detection and identification of cytokinins produced by mycorrhizal fungi. *Plant Physiology* 54: 586-588.

Díaz MSS, Álvarez CC (2009). Plant regeneration through direct shoot formation from leaf cultures and from protocorm-like bodies derived from callus of *Encyclia mariae* (Orchidaceae), a threatened Mexican orchid. *In Vitro Cellular and Developmental Bilogy-Plant* 45: 162-170.

Eymar E, Alegre J, Toribio M, López-Vela D (2000). Effect of activated charcoal and 6-benzyladenine on *in vitro*

nitrogen uptake by *Lagerstroemia indica*. *Plant Cell Tissue and Organ Culture* 63: 57-65.

Fernie AR, Willmitzer L (2001). Update on tuber formation, dormancy and sprouting: molecular and biochemical triggers of potato tuber development. *Plant Physiology* 127: 1459-1465.

Gaspar T, Kevers C, Penei C, Greppin H, Reid DM, Thorpe TA (1996). Plant hormones and plant growth regulators in plant tissue culture. *In vitro Cellular and Developmental Biology-Plant* 32: 272-289.

Haberer G, Kieber JJ (2002). Cytokinins: New insights into a classic phytohormone. *Plant Physiology* 128: 354-362.

Jackson SD (1999). Multiple signaling pathways control tuber induction in potato. *Plant Physiology* 119: 1-8.

Malmgren S (1992). Large-scale asymbiotic propagation of *Cypripedium calceolus* plant physiology from a surgeon's point of view. *Botanic Gardens Micropropagation News* 1: 59-63.

Paek KY, Murthy HN (eds) (1977). Orchid Biology: Reviews and Perspectives. Temperate Oriental *Cymbidium* species. Dordrecht: Kluwer Academic Publishers.

Pedroso MC, Pais MS (1992). Minituber production from immature seed suspension culture of *Orchis papilionacea*. *In Vitro Cellular and Developmental Biology* 28: 183-186.

Sarkar D (2008). The signal transduction pathways controlling in plant tuberization in potato: an emerging synthesis. *Plant Cell Reports* 27: 1-8.

Sezik E (1984). Orkidelerimiz. Sandoz Kültür Yayınları, pp 166.

Sgarbi E, Grimaudo M, Del Prete C (2009). *In vitro* asymbiotic germination and seedling development of *Limodorum abortivum* (Orchidaceae). *Plant Biosystems* 143: 114-119.

Skoog F, Miller CO (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology* 11: 118-130.

Stewart LS, Kane ME (2006). Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tissue and Organ Culture* 86: 147-158.

Van Waes JM, Debergh PC (1986). *In vitro* germination of some Western European Orchids. *Physiologia Plantarum* 67: 253-261.

Vesjadova H (2006). Factors affecting seed germination and seedling growth of terrestrial orchids cultured *in vitro*. *Acta Biologica Cracoviensia Series Botanica* 48: 109-113.

Vreugdenhil D, Sergeeva LI (1999). Gibberellins and tuberization in potato. *Potato Research* 42: 471-481.

Werbrouck SPO, Debergh PC (eds) (1994). Plant Cell Culture - A Pratical Approach. Applied aspects of plant regeneration (micropropagation). New York: Oxford University Press.